Fraction (31), $\eta_{sp}/c.$, 0.538, 0.478, 0.420; $[\eta]$, 0.291.

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Studies on the Conjugated Lipids

Part XIV*. Fatty Acid Constituents of Sphingomyelin from Horse Spinal Cord**

By Yasuhiko FUJINO and Takashi NEGISHI

Department of Dairy Science, Obihiro Zootechnical College, Obihiro, Hokkaido, Japan

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The chemical nature of fatty acids was studied on sphingomyelin which was prepared from horse spinal cord. Nervonic, palmitic, stearic and behenic acids were isolated from the hydrolysate of the sphingomyelin preparation. The presence of behenic acid in sphingomyelin has in this paper been reported for the first time.

It is known that several kinds of fatty acid can attach to the amino group of the shingosine moiety of shingomyelin, the structure of which is shown in Fig. 1. However, an assortment of fatty

R: Fatty alkyl group

FIG. 1. Structure of Sphingomyelin

acids in shingomyelin from a certain animal tissue seems to be almost settled, although some confusion and inconsistencies on this point are seen in the literature. This may be unavoidable

because sphingomyelin prepared by the older methods might often have been contaminated with other lipids. Nowadays, it is generally believed that sphingomyelin from brain contains lignoceric, stearic and nervonic acids as the component fatty acids¹⁻³, and that sphingomyelin from lung or spleen contains lignoceric and palmitic acids^{3,4}. The present authors undertook to elucidate the fatty acid constituents of sphingomyelin from spinal cord, since the tissue has never been studied in respect to this problem.

A pure sample of sphingomyelin was prepared from horse spinal cord, and hydrolyzed with methanolic sulfuric acid to produce methyl

* Part XIII: This Bulletin, 22, 426 (1956).

** A preliminary communication of this work has been published in the *Nature*, 184, 817 (1959).

1) P. A. Levene, *J. Biol. Chem.*, **24**, 69 (1916).

2) W. Merz, *Z. Physiol. Chem.*, **193**, 59 (1930).

3) S. J. Thannhauser and N. F. Boncoddio, *J. Biol. Chem.*, **172**, 141 (1948).

4) S. J. Thannhauser, J. Benotti and N. F. Boncoddio, *J. Biol. Chem.*, **166**, 677 (1946).

esters of fatty acids, which were divided into three fractions, 1, 2 and 3, according to their respective solubilities. Free fatty acids were isolated by saponification of each fraction, and then subjected to investigations of their physical and chemical properties.

Fatty acid from fraction 1 was proved to be nervonic acid on the basis of melting point, elementary analysis, molecular weight by titration and iodine number. Fatty acid from fraction 2 was found to consist of equal portions of palmitic and stearic acids judging from neutralization equivalent, chromatographic behavior and melting point. Fatty acid from fraction 3 was at first anticipated to be lignoceric acid, the predominant acid in the usual preparations of sphingomyelin, since this fraction yielded a larger amount of acid than the other fractions. The melting point of the material and its methyl ester, however, did not agree with that of lignoceric, but with that of behenic acid series. The neutralization equivalent, paper chromatography and the infra-red spectra indicated with certainty that the fatty acid from fraction 3 was behenic acid.

It has been shown, therefore, that sphingomyelin from horse spinal cord contain nervonic, palmitic, stearic and behenic acids as the fatty acid constituents, although the possibility is not completely excluded that some other acids may as well exist in small quantities in the preparation. Behenic acid among the four mentioned above is the one which has never been described in the literature as the principal component acid in sphingomyelin from normal animal tissue, though it has been detected in cerebroside from spleen of Gaucher's disease^{5,6} as well as in sphingomyelin from brain in a very small amount⁷.

EXPERIMENTAL

1. Preparation of Sphingomyelin*

Crude sphingomyelin prepared from the lipid fraction

* Details will be published in another communication.

5) E. Klenk, *Z. Physiol. Chem.*, **267**, 128 (1940~41).

6) A. Rosenberg and E. Chargaff, *J. Biol. Chem.*, **233**, 1323 (1958).

7) F. Rennkamp, *Z. Physiol. Chem.*, **284**, 215 (1949).

of horse spinal cord was carefully purified by treatment with alkali⁴ and successively by alumina column chromatography⁸. The purified sphingomyelin was several times recrystallized from ethyl acetate. Thus, 3 kg of fresh tissue yielded at most about 15 g of snow-white crystals of sphingomyelin. M.p. 197~198°C. $[\alpha]_D^{20} = +5.2^\circ$ ($c=0.97$ in chloroform-methanol). The preparation was practically pure as judged from the analytical data, being quite uncontaminated with glycerolipids or glycolipids. Anal. Found: P, 3.95; N, 3.46; P:N=1:2; glycerol, negligible; sugar, trace. Calcd. for $C_{45}H_{93}N_2PO_7$ (behenyl-sphingomyelin): P, 3.85; N, 3.47; P:N=1.0:2.0; glycerol, zero; sugar, zero.

2. Hydrolysis of Sphingomyelin

Six grams of pure sphingomyelin were refluxed for eight hours with 200 ml of 10% sulfuric acid in methanol. The reaction mixture was allowed to stand in an ice box overnight after hydrolysis. Large amounts of the precipitate formed on cooling were separated from the mother liquor by filtration and washed with cold methanol. The filtrate was shaken several times in a separatory funnel each time with 80 ml of petroleum ether to remove methyl esters of unsaturated fatty acids. The petroleum ether fraction was twice washed with water and concentrated to dryness by distilling under reduced pressure; weight 0.5 g (fraction 1). The precipitate, chiefly methyl esters of saturated acids, consisted of a smaller amount of yellow semisolid lump and a larger quantity of white solid material. The former was simply separated from the latter by picking up with a pincette, washed once with methanol and dried; weight 0.7 g (fraction 2). The solid material was brought to dryness as it was, to yield a rustling powder; weight 1.4 g (fraction 3).

3. Isolation of Fatty Acids

Each fraction obtained above was saponified for one hour with 30 volumes of 0.5 N potassium hydroxide in methanol. An equal volume of ether was added to the reaction mixture to precipitate the salts of fatty acids, which formed a gel on cooling. After having been filtered and washed with ether, the potassium salts were dissolved in water, removed to a separatory funnel, acidified with 2 N sulfuric acid and then shaken with ether. The ether layer was washed with water, kept with anhydrous sodium sulfate overnight and evaporated under diminished pressure. The residue obtained was recrystallized repeatedly to a constant melting point by dissolving in boiling absolute ethanol and subsequent

8) E. Klenk and F. Rennkamp, *Z. Physiol. Chem.*, **267**, 145 (1940~41).

FIG. 2. Infra-red Spectra of Behenic Acid (KBr).

a: Authentic behenic Acid

b: The acid from fraction 3

cooling in a refrigerator. Thus, fraction 1 yielded 0.1 g, fraction 20.6 g and fraction 30.9 g of free fatty acid, respectively, as a white crystalline material.

4. Identification of Fatty Acid from Fraction 1

Fatty acid from fraction 1 melted at $39\sim 40^\circ$, which suggested its possible identity with nervonic acid, m.p. 41°C . The analytical data, molecular weight by titration and iodine number were in good agreement with those of nervonic acid. *Anal.* Found: C, 78.11; H, 12.32; M.W. 360; iodine number, 65. Calcd. for $\text{C}_{24}\text{H}_{46}\text{O}_2$ (nervonic acid): C, 78.26; H, 12.56; M.W., 366; iodine number, 69.3.

5. Identification of Fatty Acid from Fraction 2

Fatty acid from fraction 2 melted at $54\sim 55^\circ\text{C}$ which apparently suggested a relationship with myristic acid, but the neutralization equivalent made it necessary to consider it a mixture of palmitic and stearic acids. The paper chromatogram⁹⁾ supported the latter suggestion; two spots on the paper clearly corresponded to the standard samples of palmitic and stearic acids, respectively. In addition, the mixture of authentic palmitic and stearic acids (1:1) gave a melting point of 55°C . These results indicate that the material from fraction 2 consists of equal parts of palmitic and stearic acids, although the analytical value of carbon in the elementary analysis was somewhat out of line. *Anal.* Found: C, 77.56; H, 12.60; M.W., 270; iodine

number, trace. Calcd. for $\text{C}_{16}\text{H}_{32}\text{O}_2$ (palmitic acid): C, 75.18; H, 12.48; M.W. 256, iodine number, zero. Calcd. for $\text{C}_{18}\text{H}_{36}\text{O}_2$ (stearic acid): C, 76.05; H, 12.67;

FIG. 3. Infra-red Spectra of Barium Behenate (KBr).

a: Authentic barium behenate

b: Barium salt of the acid from fraction 3

Dot: Band number

M.W., 284; iodine number, zero.

6. Identification of Fatty Acid from Fraction 3

Fatty acid from fraction 3 could not be identified from the elementary analysis. However, the melting point of the material, 79~80°C, and that of its methyl ester, 52.5~53.5°C, suggested it to be behenic acid. The neutralization equivalent and paper chromatography⁹⁾ supported this. *Anal.* Found: C, 76.95, H, 12.84; M.W., 342; iodine number, trace. Calcd. for $C_{22}H_{44}O_2$ (behenic acid): C, 77.58; H, 13.02; M.W., 340; iodine number, zero.

Furthermore, the infra-red spectra of this material and its barium salt were identical with those of C_{22} series, as shown in Fig. 2 and Fig. 3. It was especially striking that in the spectrum of the salt in Fig. 3 eleven bands were distinctly recognized between wave lengths 7.43 and 8.47 μ , which are characteristic of the salt of a C_{22} acid since the number of bands in this region is

9) Y. Inouye and M. Noda, *J. Agr. Chem. Soc. Japan*, **26**, 634 (1952).

equal to half the number of carbons in the straight chain-fatty acids according to Meiklejohn et al.¹⁰⁾. Thus, it is certain that the fatty acid from fraction 3 is behenic acid.

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10) R. A. Meiklejohn, R. J. Meyer, S. M. Aronovic, H. A. Schuette and V. W. Meloch, *Anal. Chem.*, **29**, 329 (1957).

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The Studies on the Action of Yeast Hexokinase upon the α - and β -Diastereomers of D-Glucose

By Hideo KATAGIRI, Kazutami IMAI and Sanehide KOMAKI

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University

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The yeast hexokinase is highly specific for α -isomer of D-glucose. The relative rate of phosphorylation of β -D-glucose, catalyzed by the purified yeast hexokinase, is observed to be 60~70 (α -D-glucose=100). The average Michaelis constants of yeast hexokinase are found to be 1.8×10^{-4} and 2.4×10^{-4} for α -D-glucose and β -D-glucose respectively, therefore the difference between the two constants is considered to be negligible.

INTRODUCTION

Although fairly stable in crystalline form, sugars undergo many transformations when dissolved in water. The interconversion between the α - and β -diastereomers of the same enatio-

morph takes place under the mildest possible condition. This phenomenon was first observed by Dubrunfaut¹⁾ who noted that the optical rotation of freshly dissolved glucose changed

1) cf. W. W. Pigman, "Chemistry of the Carbohydrate", Academic Press, 1948, p. 63.